Ischaemia/reperfusion in rat renal cortex: vesicle leakiness and Na⁺, K⁺-ATPase activity in membrane preparations

Gabriela Coux¹, Maria Mónica Elías¹ and Laura Trumper²

¹Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and ²Consejo de Investigaciones Universidad Nacional de Rosario (CIUNR), Farmacología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, (S2002LRK) Rosario, Argentina

Correspondence and offprint requests to: Laura Trumper; E-mail: ltrumper@fbioyf.unr.edu.ar

Abstract
Background. Despite the central role of Na⁺, K⁺-ATPase (NKA) in ischaemic renal injury (IRI), cortical NKA activity values during renal ischaemia remain controversial. In this study, we explore why cortical NKA activity shows such behaviour during ischaemia in rats.

Methods. Ischaemia was induced by unilateral renal artery clamping (40 min, I) followed or not by reperfusion (60 min, IR). NKA α- and β-subunit abundance was analysed by western blot. We studied the renal cortex detergent sodium dodecyl sulphate (SDS) enzymatic activation in isolated membrane preparations from control and ischaemic kidneys.

Results. NKA activity was diminished in I cortical homogenates (C = 9.3 ± 1.1, I = 4.7 ± 1.1 µmol Pi/h mg Prot, n = 4–6, *P < 0.05 versus C). This was rapidly recovered after reperfusion (IR = 9.9 ± 1.2 µmol Pi/h mg Prot). α-subunit levels were increased, while β-subunit was unchanged. At SDS 0.9 mg/ml (maximal detergent activation), the activities were indistinguishable (C = 90.5 ± 2.2, I = 91.4 ± 15.1 µmol Pi/h mg Prot). The analysis of detergent activation of NKA activity is widely used to estimate membrane leakiness in plasma membrane preparations. Our results suggest a higher population of sealed impermeable vesicles in preparations from ischaemic renal tissue.

Conclusion. The well-known effect of ischaemia on renal cell cytoskeleton could explain the observed changes in the leakiness of membrane vesicles.

Keywords: ischaemia/reperfusion; K⁺-ATPase; Na⁺; renal cortex; SDS

Introduction
Na⁺, K⁺-ATPase (NKA) is an integral membrane protein located in the basolateral membrane by direct interactions with membrane-associated cytoskeletal proteins [1]. The functional enzyme unit is a heterodimer of two subunits: α (~110 kDa, catalytic) and β (~55 kDa) [2]. Following an ischaemic insult, proximal tubular cells exhibit a disruption of the actin-based cytoskeleton [3,4], NKA dissociates from its cytoskeletal anchorage [5–7] and relocates into the apical domain in proximal tubular cells [5,8]. These
phenomena impair vectorial sodium transport across the epithelial monolayer [5,9].

Controversial cortical NKA activity values have been reported during ischaemia. Kim et al. have described decreased activity of the pump in homogenates after 120 min of ischaemia [10]. During similar periods no changes [11] or increased activity [12] of the pump was reported. We showed decreased cortical NKA activity after 40 min of ischaemia in male Wistar rats [13] but normal activity after 40 min of ischaemia followed by 1 h of reperfusion [14]. In experimental models of ischaemia, NKA activity is usually measured in preparations of isolated plasma membranes. During membrane preparation, sealed and leaky vesicles are formed. In addition, sealed vesicles could be right side out or inside out. Ouabain and ATP diffuse poorly across the membrane and have opposite sidedness of action. Ouabain-sensitive ATPase activity requires Na\(^+\), Mg\(^2+\) and ATP at the cytoplasmic face of the membranes and K\(^+\) and ouabain at the extracellular face [15]. In sealed, right-side-out vesicles, no ATPase activity would be measured, whereas this activity would be expressed in inside-out vesicles but would be unaffected by ouabain. On the other hand, ouabain-sensitive NKA activity should be fully expressed in sheets or leaky vesicles. Thus, the measured NKA activity could reflect the vesicular nature of the membrane preparations. To explore if this fact could influence NKA activity measured during ischaemia, we performed experiments in homogenates and enriched plasma membrane preparations of renal cortical tissue from control rats, and from rats that underwent ischaemia and ischaemia reperfusion. Vesicles were rendered more permeable to substrates by treatment with a detergent.

Materials and methods

Animals and treatments

Male Wistar rats (3 months, 250–350 g body weight) were anaesthetized with sodium thiopental (70 mg/kg body weight, i.p.). The right renal artery was exposed by a flank incision, and a non-traumatic vascular clamp was placed around the right renal artery for 40 min, followed (IR) or not (I).

Animals and treatments

Male Wistar rats (3 months, 250–350 g body weight) were anaesthetized with a detergent.

Materials and methods

Animals and treatments

Male Wistar rats (3 months, 250–350 g body weight) were anaesthetized with sodium thiopental (70 mg/kg body weight, i.p.). The right renal artery was exposed by a flank incision, and a non-traumatic vascular clamp was placed around the right renal artery for 40 min, followed (IR) or not (I) by 60 min of reperfusion. Total ischaemia and reperfusion were confirmed by observing the kidney surface. Sham-operated rats were used as control kidney donors (C).

Isolation of membrane fraction and NKA activity studies

A partially purified plasma membrane fraction (TPM) consisting predominantly of a mixture of basolateral and brush-border membrane vesicles from cortex was obtained as described by Boumendil-Podevin [16]. These samples were pretreated according to Forbush [17] with minor modifications (see the next section for details), and NKA activity was determined as the difference of ATPase activity in the absence of ouabain or in the presence of 1 mM ouabain and the absence of potassium as previously described [13,14,18].

Estimation of leakiness of membrane vesicles

To disrupt the vesicular structure, sodium dodecyl sulphate (SDS) was used. The plasma membrane samples (100 μl containing 30–70 μg protein) were pretreated with an equivalent volume of a buffer so the final concentrations were HEPES 17 mM, BSA 0.7%, pH 7.0 and variable concentrations of SDS: 0, 0.2, 0.4, 0.6, 0.8, 0.9, 1.2 and 1.8 mg/ml. After incubation for 10 min at 22°C, aliquots were transferred to assay tubes and NKA was determined. The percentage of leaky vesicles was estimated as follows: NKA activity (without SDS)/NKA activity (SDS − pretreated) × 100. The percentage of sealed vesicles was estimated as the difference from 100% [16]. BSA was used to buffer the free detergent concentration [17]. The optimal pretreatment condition (SDS concentration 0.9 mg/ml) was used to measure NKA activity in cortical homogenates.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

For NKA detection, samples (10 μg protein) were subjected to SDS-PAGE on polyacrylamide gels (8%) [19] and transferred onto nitrocellulose membranes as described [20]. NKA α-subunit was detected with a goat polyclonal antibody to the rabbit kidney NKA α-subunit (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) as described [13,14,21]. The primary antibody only recognized a double band that migrates near the 116-kDa marker. The split of the kidney NKA α-subunit into two bands has been previously reported [22,23]. NKA β-subunit was detected with an anti-NKA β1 (Upstate Biotechnology, Lake Placid, NY, USA, cat. 06-170) as described [21].

Statistical analysis

Results are expressed as mean ± SEM. Data were analysed using the ANOVA followed by Newman–Keuls contrasts. The 0.05 level of probability was used as the criterion of significance.

Results

NKA subunit expression and activity during ischaemia and reperfusion

NKA activity in cortical homogenates was determined under saturating conditions for sodium, potassium, magnesium and ATP. After 40 min of artery clamping, NKA activity was diminished. However, after 40 min of ischaemia and 1 h of reperfusion cortical NKA activity returned to control values (Table 1, column H, in the absence of SDS). At the same time, the abundance of α-subunit was increased while the β-subunit showed no changes (Figure 1A). As NKA α-subunit possesses the catalytic site, we presumed that the lower activity could not be attributed to changes in protein abundance but to changes in other factors affecting the measurement of the activity (e.g. vesicular nature of the membrane preparation).

Effect of ischaemia on cortical NKA latent activity

Figure 1B shows the latent activity of NKA when the membrane vesicles were rendered permeable to ATP by treatment with SDS. The specific activity of NKA in controls was increased to ~75% at SDS 0.9 mg/ml. The use of BSA as a detergent buffer, as described by Forbush [17], allowed a more relaxed working range of the SDS-membrane protein ratio. However, at concentrations >1.2 mg/ml, SDS caused a gradual decrease of the specific activity of NKA. The NKA activity of isolated plasma membranes from I differs from C in the absence and at the lowest SDS concentration checked. At SDS 0.9 mg/ml, where the maximal detergent activation is reached, activities were indistinguishable (for activity values see TPM activities in the presence of SDS in Table 1). IR values did not differ from C at any SDS concentration.

As explained [15,16], the percentage of sealed vesicles can be estimated from the degree of detergent activation (Table 1). In our case, it can be calculated for control total membranes that ~29% ((26.2/90.5) × 100) of the vesicles
are leaky and 71% are sealed. I total plasma membranes are ~15% ((13.9/91.4) × 100) leaky and 85% sealed. So the difference in the population of sealed vesicles could account for the different activities measured in I in the absence of detergent activation. This is rapidly reversed when reperfusion is allowed (see IR values in Table 1). The same analysis can be performed from the activities measured in C, I and IR homogenates, although the less purified nature of these samples may probably render less clear differences.

**Discussion**

Renal plasma membrane vesicles prepared by various methods have proved to be useful in the study of membrane phenomena at luminal or basolateral surfaces. Such studies, however, rely on the isolation of these epithelial membranes and on their homogeneity with respect to tightness and orientation of the sealed vesicles. It is well established that the NKA activity in the crude membrane fractions from a mammalian kidney is increased several fold on exposure to low concentration of detergent and that detergent activation results from increased permeability of the membranes to substrates and inhibitors [24]. Although this hypothesis has been further supported by Walter [25] and Jones [26], the complexities of detergent activation of ligand binding have led some authors to propose that the phenomenon reflects alteration of subunit interactions [27], while others have suggested changes in lipid–protein interactions or artificial enhancement of activity. These theories have been abandoned in view of the results of freeze fracture [28] and trypsin sensitivity studies [15].

A hallmark of ischaemic renal injury (IRI) is disruption of the actin-based cytoskeleton in the proximal tubule cell [3,4,6]. On the other side, the crucial importance of the actin network for the mechanical stability of cells has been reported [29]. It was also suggested that a cell’s structural strength is not solely determined by cytoskeletal composition but equally importantly by (actin) cytoskeletal architecture [30]. These two facts could suggest that during ischaemia, the mechanical stability and plasma membrane structural strength of renal cells are affected. These would certainly affect the nature of vesicle formation during cell disruption for membrane isolation.

In the present investigation, we provide evidence that during ischaemia and reperfusion total NKA activity (SDS pretreated) in renal cortex remains at control levels. However, ischaemia affected the level of SDS activation of NKA activity. During ischaemia, we found more sealed vesicles in our membrane preparations. SDS activation of NKA activity occurred in both crude homogenates and isolated TPM. Any intracellular compartment would be eliminated by the centrifugation procedure followed to obtain TPM [16]. This fact can exclude any important contribution of pre-existing intracellular vesicles in the increment in sealed vesicles found during ischaemia. We propose that the different detergent activation of NKA in ischemia could be the result of differences in the mechanical resistance of the plasma membrane due to ischemia that are put in evidence by the homogenization procedure. This could be another consequence of the well-known cytoskeletal disruption that characterizes renal ischaemia.

Ischaemic-induced acute renal failure is characterized by an impairment in water and ion reabsorption that in turn produces a reduction in glomerular filtration rate [31]. Our results show that during renal ischaemia, cortical NKA activity remains at control values, although a higher population of sealed impermeable vesicles may complicate its assessment. The preserved activity of NKA observed during I and IR reinforces the idea that impaired sodium reabsorption during IRI involves a complex interplay of factors including, among others, apical NKA redistribution [5], alterations in the expression of other sodium transporters [32] and cell detachment due to mislocalization of adhesion molecules [33].

In conclusion, the measurement of NKA activity can be affected by the vesicular nature of the membrane preparation. To guarantee its correct estimation, NKA activity studies should be performed in the presence of a detergent. In fact, a study of NKA activity/detergent concentration...
dependence is highly advisable as it would provide information regarding the vesicular state of the preparation.

Conflict of interest statement. None declared.

References

IL-1RI deficiency ameliorates early experimental renal interstitial fibrosis

Lynelle K. Jones1, Kim M. O’Sullivan1, Timothy Semple1, Michael P. Kuligowski1, Kei Fukami2, Frank Y. Ma3, David J. Nikolic-Paterson1,3, Stephen R. Holdsworth1,3 and A. Richard Kitching1,3,4

1Department of Medicine, Centre for Inflammatory Diseases, Monash University, Monash Medical Centre, Clayton, 2Vascular Division, The Baker Heart Research Institute, Danielle Alberti Memorial Centre for Diabetes Complications, Melbourne, 3Department of Nephrology and 4Department of Paediatric Nephrology, Monash Medical Centre, Clayton, Victoria, Australia

Correspondence and offprint requests to: A. Richard Kitching; E-mail: richard.kitching@med.monash.edu.au

Abstract

Background. IL-1β has the potential to promote progressive renal disease by effects on macrophage recruitment and activation or by effects mediated through tubular cell transforming growth factor (TGF)-β production, previously demonstrated in vitro.

Methods. The in vivo roles of endogenous IL-1β and its type I receptor (IL-1RI) in renal fibrosis were studied using wild-type C57BL/6 mice, IL-1β−/− and IL-1RI−/− mice with unilateral ureteric obstruction.

Results. After 7 days, IL-1RI−/− mice (IL-1α and IL-1β deficient) were protected from injury and collagen accumulation. IL-1β−/− mice demonstrated some histological protection, but no reduction in α1(1) procollagen mRNA or biochemically measured collagen accumulation. Compared with obstructed kidneys from wild-type mice, TGF-β1 mRNA was reduced in IL-1RI−/− mice (with trends to reduced TGF-β2 and TGF-β3). Expression of a downstream TGF-β effector, connective tissue growth factor, was decreased in IL-1RI−/− mice. IL-1RI−/− mice exhibited less tubulointerstitial apoptosis compared with wild-type mice. Macrophage infiltration and adhesion molecule mRNA expression was unchanged in IL-1β−/− or IL-1RI−/− mice. While TNF expression was similar to wild-type mice, IFN-γ expression was reduced in both IL-1β−/− and IL-1RI−/− mice. IL-1RI−/− mice at 14 days showed a catch-up in fibrosis compared with wild-type mice.

Conclusion. IL-1/IL-1RI interactions are profibrotic in renal fibrosis. IL-1RI−/− mice were more protected at an early stage, associated with changes in TGF-β and downstream mediators of fibrosis, but independent of the presence of infiltrating macrophages.

Keywords: IL-1; interstitial fibrosis; macrophages; obstructive uropathy; TGF-β

Introduction

Tubulointerstitial fibrosis is a common pathway to end-stage renal failure occurring after a variety of immune and inflammatory, metabolic or haemodynamic renal insults. The initiating injury, timing and persistence of the initiating insult are all important in tubulointerstitial fibrosis. However, observations from human disease and in vitro and in vivo models have defined several processes common to tubulointerstitial fibrosis induced by a variety of different insults. These include tubular injury, the infiltration of innate immune effectors, epithelial myofibroblast...